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Affinity Labeling of Catechol *O*-Methyltransferase by the Oxidation Products of 6-Hydroxydopamine

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SUMMARY

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Various polyphenols, including 6-hydroxydopamine, 6-hydroxydopa, 5-hydroxydopamine, N^1-DL-seryl-N^2(2,3,4,-trihydroxybenzyl)hydrazine (RO4-4602), pyrogallol, and 5,6-dihydroxyindole, have been found to inactivate the enzyme catechol *O*-methyltransferase irreversibly. This inactivation could be prevented by including an antioxidant in the preliminary incubation mixture or by excluding oxygen. However, catalase did not protect the enzyme from inactivation. The mechanism of 6-hydroxydopamine inactivation was investigated, and it was observed that 6-hydroxydopamine *p*-quinone the initial oxidation product of 6-hydroxydopamine, was as effective as 6-hydroxydopamine in inactivating the enzyme. However, 2-hydroxy-5-methyl-1,4-benzoquinone, which is not able to undergo a cyclization reaction to the aminochrome system as is 6-hydroxydopamine *p*-quinone, was not effective in inactivating this enzyme. This latter observation, as well as data from kinetic experiments, suggested that the aminochrome product resulting from cyclization of 6-hydroxydopamine *p*-quinone was probably the species reacting with the enzyme. This was further substantiated by the observation that 5,6-dihydroxyindole, which when air-oxidized forms a similar aminochrome system, also produced complete inactivation of the enzyme. Kinetic experiments, using adrenochrome as a model aminochrome compound, suggested that enzyme inactivation occurred by a unimolecular reaction within a dissociable complex rather than by a nonspecific bimolecular reaction. Substrate protection studies suggested that one of the active sites of the enzyme was involved in this inactivation. The results of the present experiments provide a useful model system for studying the interaction of 6-hydroxydopamine with proteins.

INTRODUCTION

2,4,5-Trihydroxyphenethylamine (6-hydroxydopamine) has been widely used as a fairly selective chemical sympathectomy agent for norepinephrine-containing nerve terminals (1-7). Despite the efforts of many investigators, the molecular mechanism of 6-hydroxydopamine degeneration of the sympathetic adrenergic neurons is unclear and remains the subject of much current research. However, it has been shown that 6-hydroxydopamine is transported by the neuronal membrane pump

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[1] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

(1, 5) and that this neuronal uptake is a prerequisite for the degenerative effect (4). All the present molecular theories for the destructive action of 6-hydroxydopamine involve in some way or another its oxidation to 6-hydroxydopamine quinone and hydrogen peroxide. Saner and Thoenen (8) proposed that 6-hydroxydopamine quinone or other oxidation products interact with important macromolecules in the adrenergic neuron, while Heikkila and Cohen (9–12) invoked the formation of superoxide anion or hydrogen peroxide as the toxic species. The hydrogen peroxide generated from the air oxidation of 6-hydroxydopamine has been observed to be the mechanism by which 6-hydroxydopamine inactivates purified dopamine β-hydroxylase (EC 1.14.2.1) (13).

The pathway for the oxidation of 6-hydroxydopamine *in vitro* has been elucidated by Wehrli *et al.* (14) and Adams *et al.* (15, 16), and was shown under physiological conditions to involve the formation of the relatively stable 6-hydroxydopamine quinone, followed by a slow cyclization to the aminochrome I, as shown in Fig. 1. Aminochrome I then rearranges to 5,6-dihydroxyindole, which can be further oxidized to aminochrome II. Recently McCreery *et al.* (17) have provided data showing an identical pathway for the oxidation of 6-hydroxydopamine *in vivo*. The experimental observations of Adams *et al.* (16) are in contradiction with the earlier proposal of Saner and Thoenen (8) that 6-hydroxydopamine quinone undergoes a 1,4 Michael addition in position 6 to give 4,6,7-trihydroxyindoline as its major degradation pathway.

Since 6-hydroxydopamine quinone and the aminochromes I and II in the oxidation pathway of 6-hydroxydopamine all contain a conjugated α-hydroxycarbonyl system (Fig. 1) similar to that found in inhibitors of the enzyme catechol *O*-methyltransferase (18, 19) (EC 2.1.1.6), it was decided to explore the possible interaction of 6-hydroxydopamine and its various oxidation products with this enzyme. If catechol

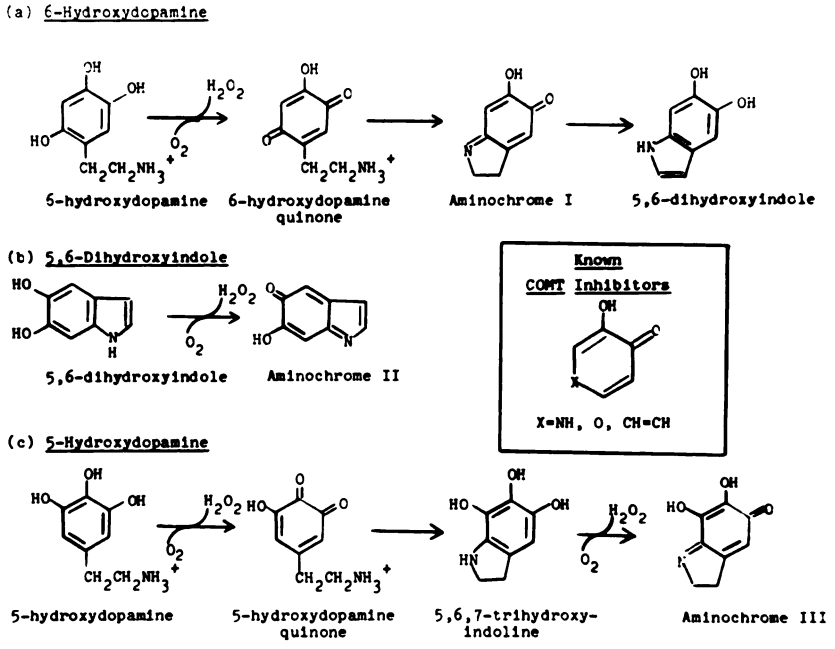


FIG. 1. *Structural relationships between oxidation products of polyphenols and known catechol O-methyltransferase (COMT) inhibitors*

For convenience of discussion, several of the compounds have been given trivial names (e.g., aminochromes I, II, and III).

O-methyltransferase has an affinity for these oxidation products, they may serve as useful affinity labeling reagents because of the known potential of these compounds to undergo nucleophilic attack (8).

In the present study it was found that 6-hydroxydopamine, as well as other polyphenols capable of generating an α-hydroxycarbonyl type of system when oxidized, irreversibly inactivate catechol *O*-methyltransferase. This appears to be a specific interaction in which the oxidation products of these polyphenols show an affinity for the active site of the enzyme and the ability to form an enzyme-ligand covalent bond. Of particular interest in this study has been the mechanism of 6-hydroxydopamine inactivation of the transferase, since this interaction could represent a model system for the interaction of 6-hydroxydopamine oxidation products with proteins. The inactivation of catechol *O*-methyltransferase by 6-hydroxydopamine is probably of little significance in the degenerative effects of this compound *in vivo*, but it has provided important insight into the mechanism of 6-hydroxydopamine interaction with a catecholamine-specific protein.

MATERIALS AND METHODS

S-Adenosyl[*methyl*-^{14}C]methionine (New England Nuclear, 55.0 mCi/mmole) was diluted to a concentration of 10 μCi/ml and stored at −20°F. *S*-Adenosylmethionine iodide (Sigma) was stored as a 0.01 M aqueous stock solution. Phosphate buffer, pH 7.60, was prepared as a 0.5 M stock solution. The following compounds were commercially available from the indicated sources: 6-hydroxydopamine, 6-hydroxydopa, 5-hydroxydopamine, catalase, and adrenochrome (Sigma); pyrogallol and dithiothreitol (Aldrich). 6-Hydroxydopamine quinone hydrobromide (14) and 2-hydroxy-5-methyl-1,4-benzoquinone (20) were obtained from Dr. R. Adams and were checked for purity. 5,6-Dihydroxyindole (21, 22) was prepared by catalytic hydrogenation of 5,6-dibenzyloxyindole (Sigma). N^1-DL-Seryl-N^2-(2,3,4-trihydroxybenzyl)hydrazine (RO4-4602) was kindly provided by Hoffmann-La Roche. Catechol *O*-methyltransferase was purified through the calcium phosphate gel step, resulting in a preparation which contained 1.34 mg of protein per milliliter with a specific activity of 47.8 nmoles of product per milligram of protein per minute with 3,4-dihydroxybenzoate as a substrate. The enzyme activity was determined using *S*-adenosyl[*methyl*-^{14}C]methionine and 3,4-dihydroxybenzoate or 3,4-dihydroxyacetophenone as substrates according to a previously described radiochemical procedure (18).

Enzyme inactivation experiments. A typical preliminary incubation mixture used to study the inactivation of catechol *O*-methyltransferase consisted of the following components: water to a final volume of 0.2 ml; magnesium chloride, 0.30 μmole; phosphate buffer, pH 7.60, 25 μmoles; varied concentrations of inhibitor; and 33.8 μg of purified enzyme preparation. In the protection experiments (Table 1) dithiothreitol (0.50 μmole), sodium metabisulfite (22.5 μmoles), or catalase (250 μg; specific activity, 11,000 units/mg) was included during the preliminary incubation. The preliminary incubation step was started by the addition of enzyme, and incubation was carried out at 37°. After the appropriate time, the samples were assayed by addition of 0.05 μCi of *S*-adenosyl[*methyl*-^{14}C]methionine, 250 nmoles of *S*-adenosylmethionine, and 500 nmoles of 3,4-dihydroxybenzoate. This resulted in final concentrations of 1.0 mM for *S*-adenosylmethionine and 2.0 mM for 3,4-dihydroxybenzoate. The assay mixtures were incubated for 5 min at 37°, and the reaction was stopped by addition of 0.10 ml of 1.0 N HCl. The assay mixture was extracted with 10 ml of toluene–isoamyl alcohol (7:3) and after centrifugation a 5-ml aliquot of the organic phase was measured for radioactivity. The results were corrected using the appropriate substrate blank. The percentage of activity remaining at any given time was calculated relative to zero-time activity. In some of the inactivation studies (Table 2; Figs. 2–6 and 8) a single large preliminary incubation mixture (3.2 ml or 1.2 ml) was prepared containing the same components at the same concentrations as

described above. Aliquots (0.2 ml) were removed at various time intervals and assayed for enzyme activity in the same manner as described above. In the anaerobic experiments (Table 2) preliminary incubation mixtures and inhibitor solutions identical with those described above were prepared, except that these were done in sealed ampoules under nitrogen and samples were removed using a syringe. Enzyme assays in these anaerobic experiments were also carried out in ampoules under nitrogen.

RESULTS

As shown in Table 1, a number of polyphenolic compounds, which are known to readily undergo air oxidation to *p*-quinoid or *o*-quinoid systems, were evaluated as irreversible inhibitors of cathechol *O*-methyltransferase. Each of these polyphenols, when oxidized, results in the formation of products which possess a conjugated α-hydroxycarbonyl system similar to that found in several classes of reversible catechol *O*-methyltransferase inhibitors (Fig. 1) (18, 19). Included in this study

TABLE 1

Effects of antioxidants and catalase on inactivation of catechol O-methyltransferase by polyphenols

The preliminary incubation mixtures were prepared as described under MATERIALS AND METHODS. Residual activity was calculated from samples containing the same additions; they were not first incubated but were assayed directly after addition of the inhibitor. Values are the averages of duplicate determinations.

Inhibitor (0.5 mM)	Additions[a]			Residual activity after 60 min at 37°
	Dithiothreitol	Sodium metabisulfite	Catalase	
				%
6-Hydroxydopamine	−	−	−	0
	+	−	−	0
	−	+	−	78
	−	−	+	8
6-Hydroxydopa	−	−	−	5
	+	−	−	2
	−	+	−	74
	−	−	+	6
5-Hydroxydopamine	−	−	−	5
	+	−	−	102
	−	+	−	97
	−	−	+	6
RO4-4602	−	−	−	7
	+	−	−	98
	−	+	−	99
	−	−	+	9
Pyrogallol	−	−	−	23
	+	−	−	101
	−	+	−	97
	−	−	+	27
5,6-Dihydroxyindole	−	−	−	0
	+	−	−	97
	−	+	−	100
	−	−	+	0

[a] When included, the concentration of dithiothreitol was 4 mM, sodium metabisulfite was 0.15 M, and catalase was 250 μg (activity, 11,000 units/mg).

were 6-hydroxydopamine, 6-hydroxydopa, and 5,6-dihydroxyindole, which are all capable of generating *p*-quinoid type oxidation products and which all produced complete inactivation of the enzyme (Table 1). Similarly, 5-hydroxydopamine, N^1-DL-seryl-N^2(2,3,4-trihydroxybenzyl)hydrazine (RO4-4602), and pyrogallol, which all generate *o*-quinoid type products when oxidized, also produced nearly complete loss of transferase activity when initially incubated with the purified enzyme. In each case this loss of enzyme activity could not be recovered after dialysis or gel filtration on Sephadex G-25, indicating that inhibition was completely irreversible. Incubation of the enzyme alone for 60 min at 37° resulted in no loss of activity. As shown in Table 1, catalase did not protect the enzyme from inactivation by any of the polyphenols tested, indicating that the hydrogen peroxide generated in the air oxidation of these compounds was not the toxic species. This was further substantiated by the observation in our laboratory that hydrogen peroxide added externally to purified catechol *O*-methyltransferase has little or no effect on the enzyme activity. In order to provide evidence that air oxidation of these polyphenols to their quinoid oxidation products was necessary to produce inactivation of catechol *O*-methyltransferase, several antioxidants were tested for their ability to protect the enzyme. As shown in Table 1, dithiothreitol could protect the enzyme from inactivation by 5-hydroxydopamine, RO4-4602, pyrogallol, and 5,6-dihydroxyindole; however, no protection was observed for 6-hydroxydopamine and 6-hydroxydopa. The latter observation is not entirely unexpected, because of the very low oxidation-reduction potential of the 6-hydroxydopamine system (15). When sodium metabisulfite, another antioxidant, was included in the preliminary incubation mixture, nearly complete protection of the enzyme from inactivation by the various polyphenols resulted. Addition of these antioxidants after preliminary incubation of the enzyme with the polyphenols did not reverse the inactivation produced by these compounds.

Further evidence to support the need for initial oxidation of these polyphenols in order to produce the inactivation of catechol *O*-methyltransferase can be seen in Table 2. In this experiment the effects of aerobic and anaerobic conditions on the inactivation process were investigated. If oxygen was excluded completely from the preliminary incubation mixtures containing the polyphenols, complete protection of the enzyme from inactivation resulted. These observations strongly support the premise that initial air oxidation of the polyphenols to quinoid oxidation products is a necessity in this inactivation process.

To explore the mechanism of this interaction further, the time course of enzyme inactivation by 6-hydroxydopamine was studied (Fig. 2). At each concentration of 6-hydroxydopamine studied, a nonlinear relationship was observed when the logarithm of percentage of activity remaining was plotted vs. preliminary incubation time. In control experiments without 6-

TABLE 2

Effects of aerobic and anaerobic conditions on inactivation of catechol O-methyltransferase by polyphenolic compounds

The standard preliminary incubation mixture consisted of inhibitor (0.56 mM), magnesium chloride (1.38 mM), phosphate buffer (pH 7.60), enzyme preparation, and water to a final volume of 1.32 ml. For the anaerobic samples a mixture of magnesium chloride, buffer, and enzyme was prepared in an ampoule under nitrogen. A solution of the inhibitor in an ampoule was prepared similarly, using deoxygenated water. At zero time an aliquot of the inhibitor was removed using a syringe and added to the enzyme mixture, and preliminary incubation was carried out. The aerobic samples were prepared similarly except that no attempt was made to exclude air. Residual activity was calculated from controls which were not first incubated but were directly assayed after addition of the inhibitor. Values are the averages of duplicate determinations.

Inhibitor (0.56 mM)	Residual activity after 60 min at 37°	
	Aerobic	Anaerobic
	%	%
6-Hydroxydopamine	0	93
6-Hydroxydopa	0	97
5-Hydroxydopamine	6	101
RO4-4602	8	96
6-Hydroxydopamine quinone	4	25
5,6-Dihydroxyindole	0	103

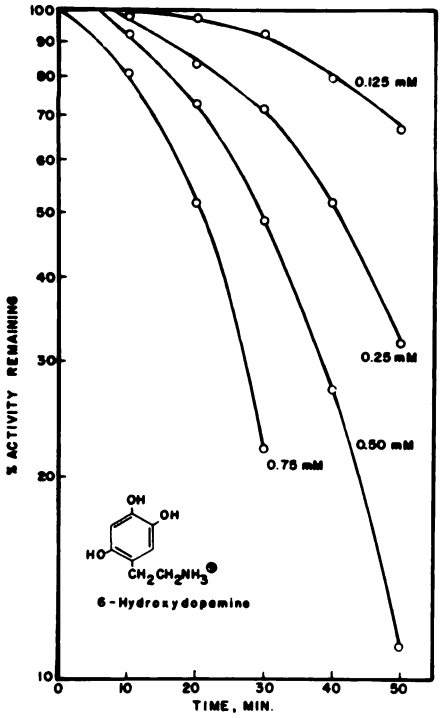


FIG. 2. *Effect of 6-hydroxydopamine on catechol O-methyltransferase activity*

Purified enzyme was first incubated with 6-hydroxydopamine, and the enzyme activity was monitored as a function of time as described in MATERIALS AND METHODS. Concentrations of the inhibitor in the preliminary incubation mixtures are noted above. Each point represents the average of duplicate determinations.

hydroxydopamine no loss of enzyme activity was observed over this time period. It is well known that 6-hydroxydopamine is extremely unstable at neutral and alkaline pH (5, 8) and that under such conditions it is rapidly oxidized to 6-hydroxydopamine quinone (15). Under the preliminary incubation conditions used in the present study (pH 7.60, 37°), nearly complete oxidation of 6-hydroxydopamine to 6-hydroxydopamine quinone would be expected to take place in the first few minutes of the preliminary incubation (15). Therefore the inactivation observed when the enzyme was first incubated with 6-hydroxydopamine probably resulted from the oxidation products generated *in situ* rather than from 6-hydroxydopamine itself. Indeed, as shown in Fig. 3, the time course for inactivation by authentic 6-hydroxydopamine quinone is remarkably similar to that observed for 6-hydroxydopamine (Fig. 2). If 6-hydroxydopamine quinone was reacting directly with the transferase via a unimolecular reaction within a dissociable complex, pseudo-first-order kinetics would be expected for such a time course of inactivation (23). Such kinetics has been observed with other affinity labeling reagents for this enzyme (23). However, as shown in Fig. 3, inactivation of the transferase by this quinone was not a pseudo-first-order process, since nonlinear plots were obtained. These results suggest that 6-hydroxydopamine quinone may not be the alkylating species; rather, the lag time observed in this inactivation may indicate that a more reactive species was being generated with time. Once formed, this species might rapidly interact with the enzyme.

To explore this possibility, it was decided to study the inactivation of catechol

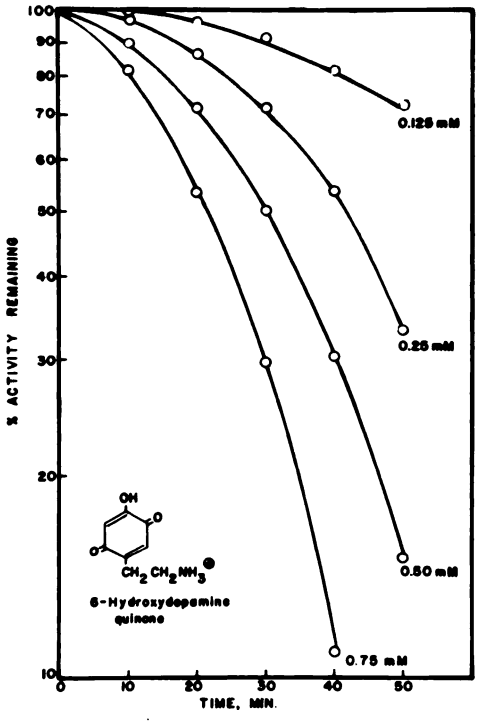
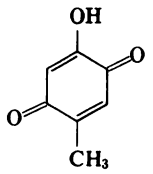


FIG. 3. *Effect of 6-hydroxydopamine quinone on catechol O-methyltransferase activity*

Enzyme activities remaining after the appropriate preliminary incubation times were determined as outlined in MATERIALS AND METHODS. Each point represents the average of duplicate determinations.

O-methyltransferase by a quinone structurally related to 6-hydroxydopamine quinone, but itself unable to undergo cyclization to the aminochrome I (Fig. 1). 2-Hydroxy-5-methyl-1,4-benzoquinone was chosen for these experiments because of its structural similarity to 6-hydroxydopamine quinone and also because it is relatively stable and cannot undergo cyclization. In Fig. 4 is shown a comparison of the time courses of inactivation produced by



2-Hydroxy-5-methyl-1,4-benzoquinone

6-hydroxydopamine quinone and 2-hydroxy-5-methyl-1,4-benzoquinone. It is readily apparent from these results that 2-hydroxy-5-methyl-1,4-benzoquinone is substantially less effective than 6-hydroxydopamine quinone as an irreversible inhibitor of catechol *O*-methyltransferase and that the resulting inactivation appears to occur by a pseudo-first-order process (linear plot of the log percentage of activity remaining vs. preliminary incubation time). This kinetics is suggestive of a mechanism involving a unimolecular reaction within a dissociable complex (23). These results would further suggest that the time course for inactivation observed in Fig. 3 does not reflect the reaction of 6-hydroxydopamine quinone with catechol *O*-methyltransferase, but rather the generation of a more reactive species, probably aminochrome I or eventually aminochrome II, and its reaction with the enzyme. The nonlinearity observed in Figs. 3 and 4 probably reflects the lag time in formation of these aminochrome systems. This conclusion is further supported by the fact that the half-life for cyclization of 6-hydroxydopamine quinone to aminochrome I has been observed to be approximately 36 min at pH 7.4 and 37°.[2]

Since the chemical conversion of 6-hydroxydopamine quinone to aminochrome I is a nonoxidative process, the elimination of oxygen from the preliminary incubation mixture would not be expected to protect the enzyme from inactivation by this quinone. Indeed, this appears to be the case, as shown in Table 2. When the preliminary incubation with 6-hydroxydopamine quinone was carried out under aerobic conditions 96% of the enzyme activity was lost, whereas under anaerobic conditions 75% of the activity was lost. Interestingly, the anaerobic conditions appear to afford some protection to the enzyme. This may reflect the fact that 5,6-dihydroxyindole, which results from rearrangement of aminochrome I, can be further air-oxidized to aminochrome II. When oxygen is excluded from the preliminary incubation of 6-hydroxydopamine quinone the eventual conversion of 5,6-dihydroxyindole to aminochrome II is retarded, which may account for the slight protection observed.

To explore further the possible involvement of 5,6-dihydroxyindole or its further oxidation products in the inactivation of catechol *O*-methyltransferase, the following experiments were carried out. 5,6-Dihydroxyindole was first tested for its ability to inactivate the enzyme, and, as shown in Table 1, this compound, like the other polyphenols, produced complete loss of activity when incubated with the enzyme for 60 min at 37°. This inactivation process could not be prevented by addition of catalase; however, inclusion of dithiothreitol or sodium metabisulfite afforded complete protection of the enzyme. Similarly, as shown in Table 2, if oxygen was excluded from the preliminary incubation mixture no inactivation by 5,6-dihydroxyindole was observed. These results would support the conclusion that the oxidation of 5,6-dihydroxyindole to the aminochrome II is necessary in this inactivation of the enzyme. In Fig. 5 are shown the time courses for inactivation of catechol *O*-methyltransferase by 5,6-dihydroxyindole. If 5,6-dihydroxyindole was added directly to the enzyme, a nonlinear relationship was observed between log percentage of activity remaining and preliminary incubation time. This apparent lag in inactivation

[2] R. Adams, personal communication.

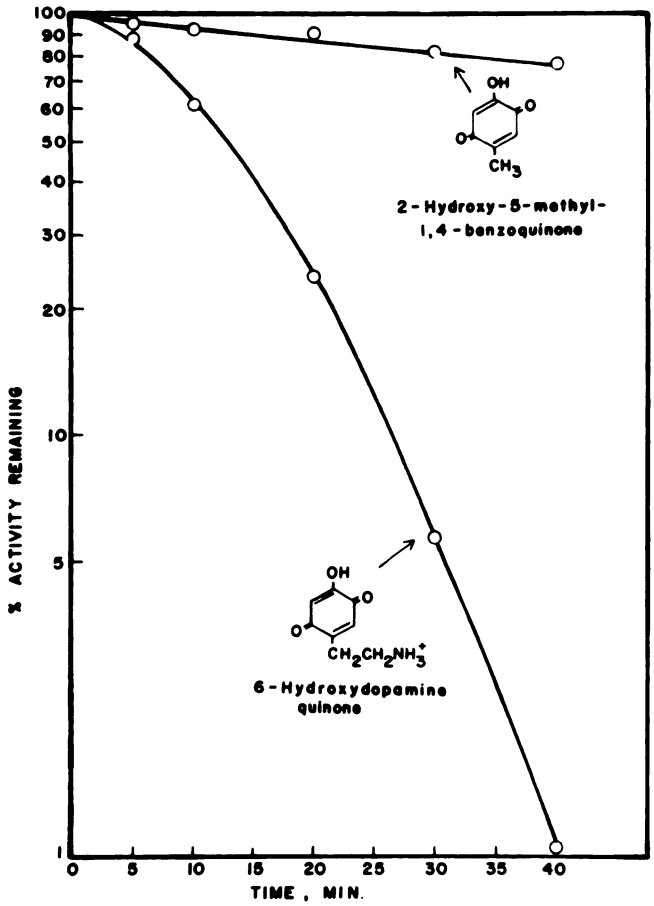
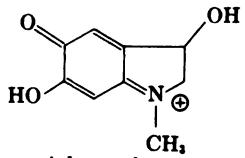


FIG. 4. *Effect of 6-hydroxydopamine quinone and 2-hydroxy-5-methyl-1,4-benzoquinone on catechol O-methyltransferase activity*

Inhibitor concentrations in the preliminary incubation mixture were 1.25 mM. After the appropriate preliminary incubation times enzyme activities remaining were determined as outlined in MATERIALS AND METHODS. Each point represents the average of duplicate determinations.

indicates that the rate of oxidation of the indole is slow. This lag time could be eliminated by the initial air oxidation of 5,6-dihydroxyindole to aminochrome II before the preliminary incubation step in phosphate buffer, pH 7.60. When an aliquot of the 5,6-dihydroxyindole–phosphate buffer mixture was added to the enzyme, a linear relationship between log percentage of activity remaining and preliminary incubation time was observed (Fig. 5).

In an effort to explore in more detail the kinetics of the reaction between catechol *O*-methyltransferase and these aminochrome compounds, the time course for inactivation of the enzyme by adrenochrome was studied. Adrenochrome has the advantages that it is a relatively stable aminochrome and it does not have to be generated *in situ*. The time courses for inactivation of the transferase by adrenochrome are shown in Fig. 6. With each concentration of inhibitor studied the plots were linear,



Adrenochrome

indicating that the reactions were first-order with respect to active enzyme remaining. In order to provide evidence that the inactivation of catechol *o*-methyl-

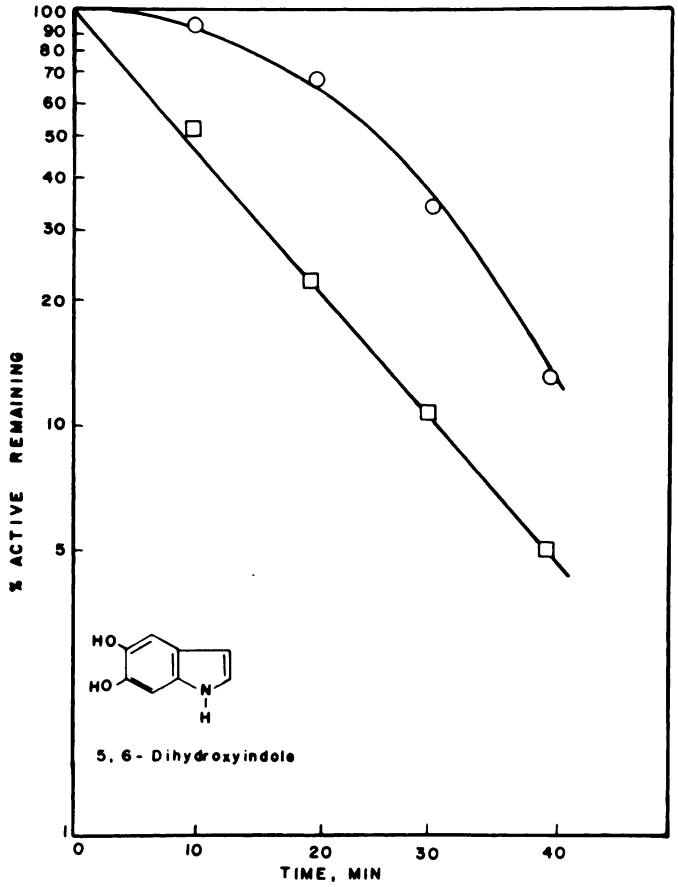


FIG. 5. *Effect of 5,6-dihydroxyindole on catechol O-methyltransferase activity*

O——O, 5,6-dihydroxyindole (0.25 mM) was added directly to a preliminary incubation mixture containing purified catechol *O*-methyltransferase, and enzyme activities were determined at the appropriate times by removing aliquots. □——□, a solution of 5,6-dihydroxyindole (5.0 mM) in phosphate buffer, pH 7.60, was incubated at 37° for 20 min, during which time it was air-oxidized to aminochrome II. An aliquot of this aminochrome II solution was added to a preliminary incubation mixture containing purified enzyme, and incubation was carried out at 37°. At the appropriate times aliquots were removed and enzyme activities were determined by the methods described in the text. Points represent the averages of duplicate determinations.

transferase by adrenochrome proceeds via a unimolecular reaction within a dissociable complex rather than a nonspecific bimolecular reaction, the rates of enzyme inactivation as a function of inhibitor concentration were investigated (Fig. 6) and evaluated as follows. The model for this type of inactivation is shown in Eqs. 1 and 2, where $E \cdot I$ is the reversible complex, $E\text{-}I$ the inactive enzyme, k_2 the first-order rate constant, and K_I the steady-state constant of inactivation ($K_I = [E][I]/[E \cdot I]$) (23, 24).

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} E\text{-}I \qquad (1)$$

$$\frac{1}{K_{app}} = \frac{K_I}{k_2[I]} + \frac{1}{k_2} \qquad (2)$$

Evidence to support the inactivation pathway shown in Eq. 1 can be obtained if a linear relationship is observed between the reciprocal of the pseudo-first-order rate constants ($1/K_{app}$) and the reciprocal of the inhibitor concentration ($1/[I]$). When the apparent first-order rate constants were calculated from the data shown in Fig. 6 and the reciprocals were plotted vs. the inhibitor concentrations, a linear relationship was observed (Fig. 7). Using Eq. 2 (23, 24), a steady-state constant of inactiva-

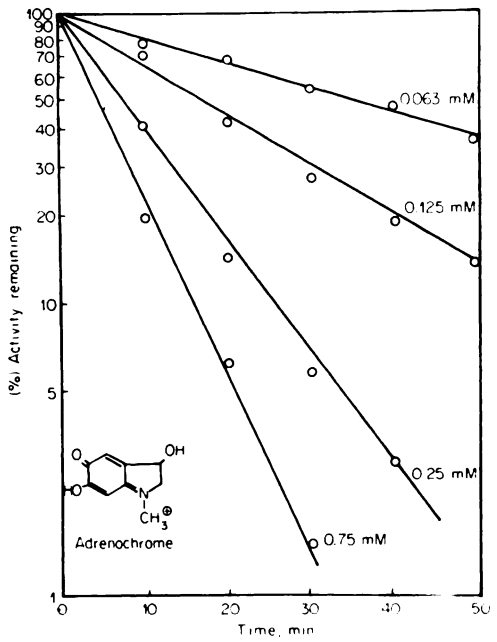


FIG. 6. *Effect of adrenochrome on catechol O-methyltransferase activity*

Preliminary incubation mixtures containing purified enzyme and varying concentrations of adrenochrome were prepared as described in MATERIALS AND METHODS. Enzyme activities were determined after the appropriate preliminary incubation times. Each point represents the average of duplicate determinations.

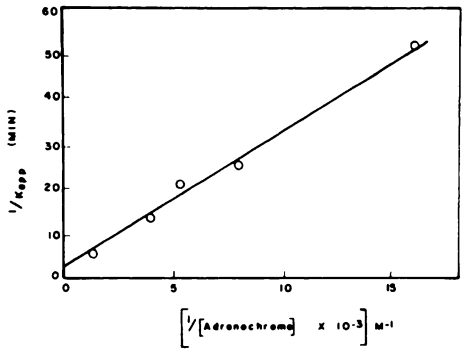


FIG. 7. *Double-reciprocal plots of pseudo-first-order rate constants of inactivation vs. inhibitor concentrations*

The pseudo-first-order rate constants of inactivation were calculated from the data shown in Fig. 6. Kinetic constants (k_2 and K_I) were calculated from the y intercept and the slope, respectively, using the least-squares method (23, 24).

tion, K_I = 2.16 ± 1.21 mM, and a first-order rate constant at saturation, k_2 = 0.66 ± 0.30 min^{-1}, were calculated for the adenochrome reaction with catechol *O*-methyltransferase. These observations provide strong evidence that the inactivation of the enzyme by adrenochrome occurs within a dissociable complex, probably involving the active site of the enzyme.

Further evidence to support the involvement of one of the active sites of catechol *O*-methyltransferase in the inactivation produced by 6-hydroxydopamine or adrenochrome comes from the protection experiments shown in Fig. 8. If the preliminary incubation of enzyme with 6-hydroxydopamine is performed in the presence of *S*-adenosylmethionine (0.1 mM), the inactivation is greatly diminished. The protection of the enzyme by *S*-adenosylmethionine is of particular interest, since this process appears to be saturable. As shown in Fig. 9, concentrations of *S*-adenosylmethionine above approximately 0.2 mM did not provide additional protection of the enzyme from inactivation by 6-hydroxydopamine. The concentration range of *S*-adenosylmethionine (1–200 μM) which produced maximum changes in the degree of protection in this system is quite similar to the concentration range which produced the greatest changes in the velocity of the enzyme reaction (K_m = 120 μM). Results similar to those with *S*-adenosylmethionine were obtained with *S*-adenosylhomocysteine, a potent product inhibitor of the catechol *O*-methyltransferase reaction. As shown in Fig. 9, *S*-adenosylhomocysteine partially protected the enzyme from inactivation by 6-hydroxydopamine. Again this appears to be a saturable process, suggesting a mechanism similar to that for *S*-adenosylmethionine. Methionine and methionine *S*-methylsulfonium iodide at concentrations up to 5 mM did not protect the enzyme from inactivation, indicating that the protection produced by *S*-adenosylmethionine and *S*-adenosylhomocysteine involved specific binding of those compounds to the active site of the enzyme.

Protection experiments similar to those described above for 6-hydroxydopamine were also carried out for the adrenochrome inactivation of catechol *O*-methyltransfer-

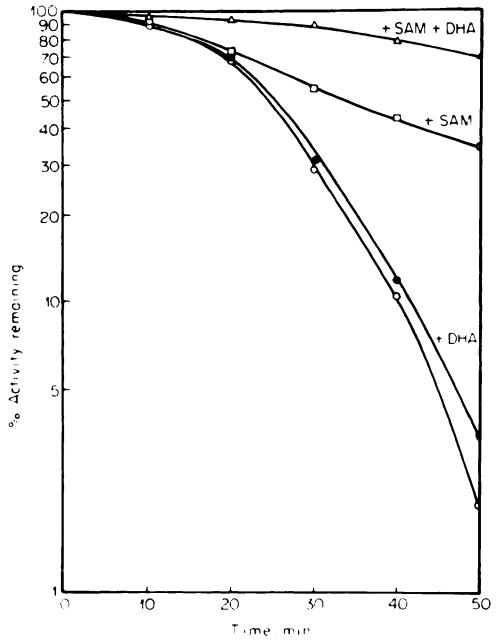


FIG. 8. *Effect of S-adenosylmethionine (SAM) and 3,4-dihydroxyacetophenone (DHA) on inactivation of catechol O-methyltransferase by 6-hydroxydopamine*

O——O, 6-hydroxydopamine only (0.75 mM); ●——●, 6-hydroxydopamine (0.75 mM) plus 3,4-dihydroxyacteophenone (2.5 mM); □——□, 6-hydroxydopamine (0.75 mM) plus *S*-adenosylmethionine (0.1 mM); Δ——Δ, 6-hydroxydopamine (0.75 mM) plus 3,4-dihydroxyacetophenone (2.5 mM) and *S*-adenosylmethionine (0.1 mM). After the appropriate preliminary incubation times enzyme activities were determined as outline in MATERIALS AND METHODS. During the assay no additional catechol substrates were added to those preliminary incubation mixtures that already contained 3,4-dihydroxyacetophenone. Each point is the average of duplicate determinations.

ase. Again partial protection by *S*-adenosylmethionine was observed. In addition, it was possible to determine the effect of *S*-adenosylmethionine on the first-order rate constant (k_2) for the reaction of adrenochrome and catechol *O*-methyltransferase. When the inactivation of the enzyme by adrenochrome was carried out in the presence of *S*-adenosylmethionine (0.1 mM) a 10-fold decrease in the first-order rate constant (k_2) for inactivation was observed, whereas the K_I remained essentially unchanged. This observation would strongly support the proposal that when *S*-adenosylmethionine binds to catechol *O*-methyltransferase the enzyme undergoes a conformational change which decreases either the accessibility or the nucleophilicity of the amino acid moiety which is being modified.

Since the aminochromes I and II, which are the proposed active intermediates in this inactivation by 6-hydroxydopamine, are α-hydroxycarbonyl compounds, catechol substrates would also be expected to protect the enzyme from inactivation. Such α-hydroxycarbonyl compounds have previously been shown to be competitive inhibitors of catechol *O*-methyltransferase when 3,4-dihydroxybenzoate or 3,4-dihydroxyacetophenone was the variable substrate (18, 19). When 3,4-dihydroxyacetophenone (2.5 mM) was included in the preliminary incubation mixture containing 6-hydroxydopamine, only slight protection of the enzyme was observed (Fig. 8). However, if both *S*-adenosylmethionine (0.1 mM) and 3,4-dihydroxyacetophenone (2.5 mM) were included in the preliminary incubation mixture, substantial protection was observed. The protection observed when both substrates were present in the preliminary incubation mixture was much greater than the protection produced by *S*-adenosylmethionine alone or expected from a simple combination of the two substrates. A similar observation has recently been made in this laboratory for a series of *N*-haloacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine affinity labeling reagents, which show a similar protection pattern.[3]

DISCUSSION

In the present study it was found that various polyphenolic compounds, including 6-hydroxydopamine, will irreversibly inactivate catechol *O*-methyltransferase. The two general classes of polyphenols used in this study were (a) compounds capable of generating *p*-quinoid type oxidation products (e.g., 6-hydroxydopamine, 6-hydroxydopa, and 5,6-dihydroxyindole) and (b) compounds capable of generating *o*-quinoid type oxidation products (e.g., 5-hydroxydopamine, RO4-4602, and pyro-

[3] R. T. Borchardt and D. Thakker, unpublished observations.

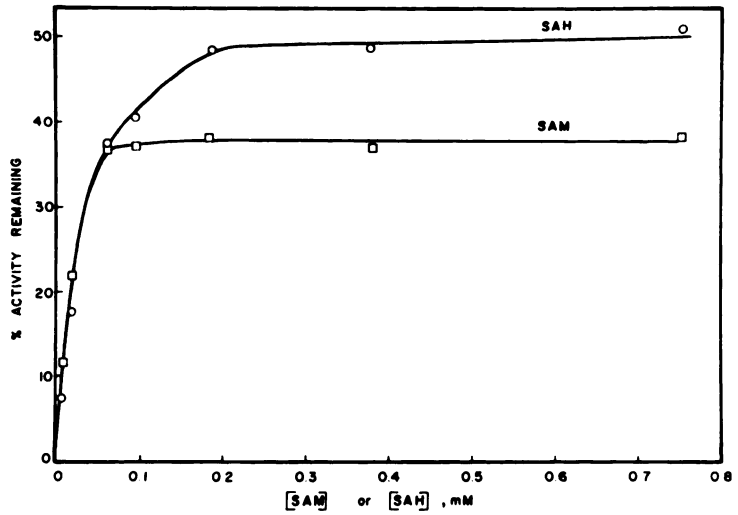


FIG. 9. *S-Adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) protection of catechol O-methyltransferase from inactivation by 6-hydroxydopamine.*

Preliminary incubation mixtures prepared as described in the text, containing purified enzyme, 6-hydroxydopamine (0.75 mM), and varying concentration of *S*-adenosylmethionine (□——□) or *S*-adenosylhomocysteine (○——○), were incubated at 37°, and aliquots were removed at zero time (controls) and 40 min. These were checked for enzyme activities as described in MATERIALS AND METHODS. Each point is the average of duplicate determinations. The percentage of activity remaining is the enzyme activity left after 40 min of preliminary incubation as compared to controls.

gallol). In each class of compounds the resulting air oxidation products possess conjugated α-hydroxycarbonyl systems similar to those found in known inhibitors of catechol *O*-methyltransferase (Fig. 1). Therefore these oxidation products would be expected to have an affinity for the active site of this enzyme and to produce its inactivation by the formation of an enzyme-ligand covalent bond. Similar inactivation of the transferase by nonspecific alkylating reagents (25, 26) and specific affinity labeling reagents (23) has been observed. In support of an alkylation mechanism for the inactivation of catechol *O*-methyltransferase by these polyphenols, Creveling and co-workers[4] have recently observed that labeled 6-hydroxydopamine is incorporated into catechol *O*-methyltransferase in a ratio of 1 molecule of 6-hydroxydopamine per molecule of enzyme. This incorporation appears to take place at one of the two sulfhydryl groups proposed to be at the active site of this enzyme (26), since if the enzyme is first treated with *N*-ethylmaleimide and then with labeled 6-hydroxydopamine, no incorporation of the latter compound is observed.

In an effort to elucidate the mechanism of this inactivation of catechol *O*-methyltransferase by 6-hydroxydopamine, this study has provided the following results. (a) Air oxidation of 6-hydroxydopamine was necessary to produce this inactivation. (b) Hydrogen peroxide was not the species toxic to the enzyme. (c) Authentic 6-hydroxydopamine quinone produced inactivation similar to that of 6-hydroxydopamine, whereas 2-hydroxy-5-methyl-1,4-benzoquinone, which could not be converted to the aminochrome I, was substantially less effective. (d) Time courses of inactivation for 6-hydroxydopamine and 6-hydroxydopamine quinone were nonlinear, suggesting that the aminochrome I, which forms by cyclization of 6-hydroxydopamine quinone, may be a more reactive species toward the enzyme. (e) 5,6-Dihydroxyindole, a further product in the oxidation of 6-hydroxydopamine, was also effective in inactivating the enzyme as long as it could be further air-oxidized to the aminochrome II. (f) Adrenochrome, a rela-

[4] C. R. Creveling, personal communication.

tively stable aminochrome type compound, was found to inactivate the enzyme rapidly, and the kinetics of inactivation suggested a unimolecular reaction within a dissociable complex rather than a nonspecific biomolecular reaction. (g) Substrate protection studies suggested that an amino acid residue at the active site of the transferase was being modified. These experimental observations would support an alkylation mechanism for the inactivation of catechol *O*-methyltransferase by 6-hydroxydopamine as depicted in Fig. 10. Of particular interest is the fact that the alkylating species in this system appears to be the aminochromes I and II or other oxidation products, rather than 6-hydroxydopamine quinone. This quinone had previously been proposed to be the reactive species in the model studies of Saner and Thoenen (8) on bovine serum albumin.

With the polyphenols which produce *o*-quinoid oxidation products (pyrogallol and RO4-4602) the mechanism of inactivation may involve direct alkylation of the resulting *o*-quinones. In proposing this direct alkylation of the *o*-quinone system, one must consider the apparent low reactivity of 6-hydroxydopamine quinone and 2-hydroxy-5-methyl-1,4-benzoquinone with catechol *O*-methyltransferase. However, an *o*-quinone, being an extremely electron-deficient species, may undergo intermolecular nucleophilic attack much more readily than a similar *p*-quinone system. In fact, R. Adams and his co-workers[2] have observed that dopamine *o*-quinone undergoes nucleophilic attack by aniline, glutathione, and cysteine much faster than it undergoes intramolecular cyclization to 5,6-dihydroxyindoline. For 5-hydroxydopamine inactivation of catechol *O*-methyltransferase, a similar direct alkylation of 5-hydroxydopamine quinone is possible. An alternative explanation is the reaction of the enzyme with aminochrome III, which would arise from the oxidation of 5,6,7-trihydroxyindoline. 5,6,7-Trihydroxyindoline would be generated from an intramolecular 1,4 Michael addition reaction on 5-hydroxydopamine quinone.

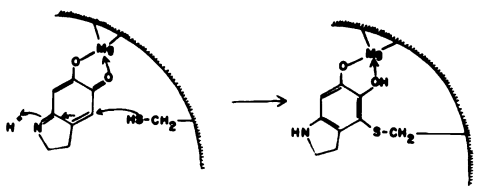


FIG. 10. *Possibile mechanism for irreversible inhibition of catechol O-methyltransferase by aminochrome I*

Of some general interest is the irreversible inhibition of catechol *O*-methyltransferase by RO4-4602, which is also a dopa decarboxylase inhibitor used in combination with L-dopa in the treatment of Parkinson's disease (27, 28). Baldessarini and Greiner (29) recently reported that RO4-4602 is a potent noncompetitive inhibitor of catechol *O*-methyltransferase and suggested that the beneficial properties of this compound in L-dopa therapy may be a result of its ability to inhibit the enzyme *in vivo*. The present observations on the inhibitory properties of RO4-4602 toward catechol *O*-methyltransferase would support this conclusion.

The results obtained in this study should be considered as a possible model system for the interaction of 6-hydroxydopamine and other polyphenols with macromolecules. The inactivation of catechol *O*-methyltransferase *in vitro* by 6-hydroxydopamine reported here is probably of little significance, if any, in the degenerative effects of this compound *in vivo*. However, this system has provided new insight into the mechanism by which 6-hydroxydopamine and related compounds can interact with macromolecules. It has also shown that because of certain structural features of the oxidation products of 6-hydroxydopamine (e.g., the conjugated α-hydroxycarbonyl system), these molecules may have a built-in specificity for proteins which normally have an affinity for catecholamines.

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REFERENCES

1. Ungerstedt, U. (1971) in *6-Hydroxydopamine and Catecholamine Neurons* (Malmfors, T. & Thoenen, H., eds.), pp. 315-332, North Holland Publishing Co., Amsterdam.
2. Porter, C. C., Totaro, J. A. & Stone, C. A. (1963) *J. Pharmacol. Exp. Ther.*, **140**, 308-316.
3. Laverty, R., Sharman, D. F. & Vogt, M. (1965) *Br. J. Pharmacol. Chemother.*, **24**, 549-560.
4. Malmfors, T. & Sachs, C. (1968) *Eur. J. Pharmacol.*, **3**, 89-92.
5. Thoenen, H. & Tranzer, J. P. (1968) *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.*, **261**, 271-288.
6. Taylor, K. M. & Laverty, R. (1972) *Eur. J. Pharmacol.*, **17**, 16-24.
7. Uretsky, N. J. & Iversen, L. L. (1970) *J. Neurochem.*, **17**, 269-278.
8. Saner, A. & Thoenen, H. (1971) *Mol. Pharmacol.*, **7**, 147-154.
9. Heikkila, R. & Cohen, G. (1971) *Science*, **172**, 1257-1258.
10. Heikkila, R. & Cohen, G. (1972) *Experientia*, **28**, 1197-1198.
11. Heikkila, R. & Cohen, G. (1972) *Mol. Pharmacol.*, **8**, 241-248.
12. Heikkila, R. & Cohen, G. (1973) *Science*, **181**, 456-457.
13. Aunis, D., Miras-Portugal, M. T. & Mandel, P. (1973) *Biochem. Pharmacol.*, **22**, 2581-2589.
14. Wehrli, P. A., Pigott, F., Fischer, U. & Kaiser, A. (1972) *Helv. Chim. Acta*, **55**, 3057-3061.
15. Adams, R. N., Murrill, E., McCreery, R., Blank, L. & Karolczak, M. (1972) *Eur. J. Pharmacol.*, **17**, 287-292.
16. Blank, C. L., Kissinger, P. T. & Adams, R. N. (1972) *Eur. J. Pharmacol.*, **19**, 391-394.
17. McCreery, R. L., Dreiling, R. & Adams, R. N. (1974) *Brain Res.*, **73**, 15-21.
18. Borchardt, R. T. (1973) *J. Med. Chem.*, **16**, 377-382, 382-387, 581-583.
19. Borchardt, R. T. & Huber, J. A. (1975) *J. Med. Chem.*, **18**, 120-122.
20. Woodward, R. B., Sondheimer, F., Taub, D., Heusler, K. & McLamore, W. M. (1952) *J. Am. Chem. Soc.*, **74**, 4223-4251.
21. Benigni, J. D. & Minnis, R. L. (1965) *J. Heterocycl. Chem.*, **2**, 387-392.
22. Harley-Mason, J. (1953) *J. Chem. Soc.*, 200-203.
23. Borchardt, R. T. & Thakker, D. (1973) *Biochem. Biophys. Res. Commun.*, **54**, 1233-1239.
24. Petra, P. H. (1971) *Biochemistry*, **10**, 3163-3170.
25. Axelrod, J. & Tomchick, R. (1958) *J. Biol. Chem.*, **233**, 702-705.
26. Morris, N. D., McNeal, F. & Creveling, C. R. (1973) *Abstr. 8th Middle Atlantic Regional Meet. Am. Chem. Soc. (Washington, D. C.)*, 60.
27. Bartholini, F. & Pletscher, A. (1968) *J. Pharmacol. Exp. Ther.*, **161**, 14-20.
28. Barbeau, A., Gillo-Joffroy, L. & Mars, H. (1971) *Clin. Pharmacol.Ther.*, **12**, 353-359.
29. Baldessarini, R. J. & Greiner, E. (1973) *Biochem. Pharmacol.*, **22**, 247-256.